Trans-10, Cis-12 Conjugated Linoleic Acid Inhibits Prolactin-Induced Cytosolic NADP⁺-Dependent Isocitrate Dehydrogenase Expression in Bovine Mammary Epithelial Cells

Wenjing Liu, Stephanie C. Degner, and Donato F. Romagnolo*

Laboratory of Mammary Gland Biology, Department of Nutritional Sciences, University of Arizona, Tucson, AZ 85721

Abstract
Conjugated linoleic acid (CLA) has been found to exert beneficial effects on lipid profile and repress de novo fatty acid synthesis in mammary gland during lactation. However, the underlying mechanisms responsible for the antilipogenic effects of CLA have not been established. The cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDH1) plays a critical role in cholesterol and fatty acid biosynthesis by providing reducing equivalents as NADPH. In previous studies, we documented that the expression of IDH1 in bovine mammary epithelium was modulated by regulators of mammary differentiation and metabolic effectors. In this study, we investigated the short-term effects of prolactin (PRL) and CLA on IDH1 expression in BME-UV bovine mammary epithelial cells. In time-course experiments, we found that the treatment with PRL for 60 and 90 min elicited a significant increase in IDH1 transcript levels. Conversely, the cotreatment of BME-UV cells with PRL plus a CLA mixture for 90 min prevented the accumulation of IDH1 mRNA induced by PRL. In addition, we found that the trans-10, cis-12 CLA, but not the cis-9, trans-11 CLA isomer, inhibited basal- and PRL-induced IDH1 mRNA expression. The inhibitory effects of the trans-10, cis-12 CLA isomer on PRL-induced IDH1 expression accumulation were confirmed by quantitative real time PCR and western-blotting analysis. We propose that the inhibitory effects of CLA on milk fat synthesis in mammary epithelial cells may derive, at least in part, from repression of IDH1 expression. J. Nutr. 136: 2743–2747, 2006.

Introduction
Conjugated linoleic acid (CLA) refers to a naturally occurring mixture of positional and geometric isomers of linoleic acid with conjugated double bonds produced by bacterial biohydrogenation in the ruminant gut (1). CLA is widely found in many foods, including dairy products, meats, and certain vegetable products (2). The cis-9, trans-11 (c9, t11) and trans-10, cis-12 (t10, c12) CLA are the major isomers in the commercially available CLA mixtures, whereas other isomers are also present at lower concentrations (3,4). It has been reported that exogenous CLA reduced bovine milk fat concentration and yield by inhibiting de novo fatty acid synthetase (FAS), and several lipogenic genes including acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS), and Δ-9-desaturase (14). Despite the wealth of data documenting the antilipogenic effects of CLA both in animals and humans, the mechanisms by which CLA alters lipid metabolism are not clearly defined. A key enzyme involved in fatty acid synthesis is the cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDH1), which provides reducing equivalents in the form of NADPH. The IDH1 enzyme generates the primary source of NADPH required for de novo fatty acid synthesis in the bovine mammary gland during lactation (15,16). The crucial role of IDH1 in lipid metabolism has also been demonstrated by Koh et al. (17) using 3T3-L1 cells

1 Abbreviations used: CLA, conjugated linoleic acid; c9, t11 CLA, cis-9, trans-11 conjugated linoleic acid; CLAmix, mixture of conjugated linoleic acid isomers; IDH1, cytosolic NADP⁺-dependent isocitrate dehydrogenase; PRL, prolactin; t10, c12 CLA, trans-10, cis-12 conjugated linoleic acid.
2 To whom correspondence should be addressed. E-mail: donato@u.arizona.edu.
and transgenic mice overexpressing IDH1 (17). Recently (18), we reported on the regulation of IDH1 expression in bovine mammary tissue and in an established mammary epithelial cell line. However, no information regarding the regulation of IDH1 expression by CLA is available.

Materials and Methods

Cell culture. The BME-UV bovine mammary epithelial cell line was kindly provided by Dr. Politis (University of Vermont, Burlington, Vermont). BME-UV cells were maintained in DMEM supplemented with 10% fetal calf serum at 37°C and 5% CO2 atmosphere and cells were routinely passaged every 6–7 d by washing with Dulbecco's PBS followed by trypsinization.

Reagents and chemicals. Bovine prolactin (PRL) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program (isolated from bovine pituitary gland, 90–95% monomeric, lot no. AF7170E). DMEM was obtained from Sigma Chemical, fetal calf serum was purchased from Hyclone Laboratories. The mixture of CLA isomers (CLAmix) was obtained from Sigma and consisted of t9, c11 and c9, t11 (50%), t10, c12 (40%), and c10, t12 (10%). Purified (~98% purity) c9, t11 and t10, c12 CLA isomers were obtained from Matreya. TriReagent was purchased from Molecular Research Center. Random hexamer primers, Moloney murine leukemia virus reverse transcriptase, and RNase inhibitor were purchased from Life Technologies. Reverse transcription buffer was obtained from Ambion. Vent DNA polymerase was purchased from New England Biolabs. The antibody against the mammary IDH1 was kindly provided by Dr. McAlister-Henn (University of Texas Health Science Center, San Antonio, TX).

Semi quantitative RT-PCR. Optimization of conditions for amplification of IDH1 are described elsewhere (18). Briefly, oligonucleotides used to amplify the IDH1 fragment (384 bp) were (forward) 5'-GTCTGTGGTAGAGCTGCAAGG-3' and (reverse) 5' -CATAAGCAGACCTATG-3'. PCRs were performed using Vent DNA polymerase. The authenticity of the IDH1 product was confirmed by direct sequencing and BLAST analysis against deposited sequences in the Genbank database. Ribosomal 18S RNA was also amplified to control for PCR conditions and equal loading. Relative levels of IDH1 were estimated by Kodak 1D Image (Eastman Kodak) analysis and corrected for expression of the control RNA.

Real-time PCR. cDNA was prepared by reverse transcription of sample RNA using the Bio-Rad iScript cDNA kit (Bio-Rad Laboratories) in a reaction volume of 40 μL. DNA standards were prepared from PCR amplicons purified using the QIAQuick PCR purification kit (Qiagen). Product concentrations and the quantity of cDNA in unknown samples were determined as previously described (19). Real-time PCR was performed using the Bio-Rad MyiQ Real-Time Single Color PCR Detection system in Bio-Rad 96-well plates in a 25-μL volume. The PCR program was as follows: 95°C initial denaturation for 3 min, followed by 45 cycles of 94°C for 15 s, 57°C for 30 s, 72°C for 1 min, 55°C for 1 min, and holding at 4°C. A 1-μL sample cDNA or standard was added to 24 μL of reaction mix in the wells. Oligonucleotides used to amplify IDH1 (149 bp) were (forward) 5'-CAAGGCGGGTCAGTGTAAG-3' and (reverse) 5'-TGTCGTTGGTGGCATCG-3'.

Western blotting. Western blotting analysis for IDH1 was performed as described previously (18). Immunoblotting was carried out with rabbit polyclonal antibody against IDH1. Normalization of western blots was confirmed by incubating immunoblots with β-actin antibody-1 (Oncogene Research Products). The immunocomplexes were detected by enhanced chemiluminescence (Amersham).

Data analysis. Fold-changes in expression of IDH1 mRNA are presented as means ± SEM. Significance (P < 0.05) of the differences between means was determined by t test or one-way ANOVA followed by Fisher's protected least significant difference test.

Results

In a recent study (18), we found that the long-term treatment (72 h) with PRL stimulates the expression of IDH1. To evaluate the short-term effects of PRL on IDH1 expression, BME-UV cells were cultured in basal medium (DMEM) or DMEM supplemented with PRL (2.0 mg/L) for 15, 30, 60, and 90 min. Compared with the basal expression measured in cells cultured in DMEM, IDH1 mRNA levels increased (P < 0.05) 94 and 122% in cells treated with PRL, respectively, for 60 and 90 min (Fig. 1). Conversely, PRL had no effect at earlier time points (15 and 30 min).

Because CLA has been shown to repress milk fat synthesis both in animals and humans (5,7), we investigated whether or not CLA influenced basal and PRL-induced IDH1 expression. We treated BME-UV cells with 2.0 mg/L PRL, 20 or 40 μmol/L CLAmix, or a combination of 2.0 mg/L PRL plus 20 or 40 μmol/L CLAmix for 90 min. For these experiments, we used a CLAmix containing c9, c11 and c9, t11 (50%), t10, c12 (40%), and c10, t12 (10%) CLA isomers. Treatment with 40 μmol/L CLAmix tended to reduce (P < 0.10) basal IDH1 mRNA levels, whereas the cotreatment with the CLAmix plus PRL completely abolished the stimulatory effects of PRL (Fig. 2A,B). These cumulative data suggest that CLA prevents the rapid PRL-induced stimulation of IDH1 mRNA expression.

Because distinct biological effects have been documented for various CLA isomers (20), we tested whether or not c9, t11 CLA and t10, c12 CLA differentially regulated IDH1 expression. We cultured BME-UV cells for 90 min in DMEM, DMEM supplemented with 40 μmol/L c9, t11 CLA or t10, c12 CLA in the presence or absence of 2.0 mg/L PRL and measured IDH1 mRNA levels. We observed that the treatment with the t10, c12 CLA isomer decreased (P < 0.05) both basal and PRL-induced IDH1 mRNA expression. In contrast, the c9, t11 CLA isomer had no significant effects on basal and PRL-stimulated IDH1 expression.

Figure 1 PRL induces IDH1 mRNA in bovine mammary epithelial cells. BME-UV cells were cultured in DMEM or DMEM supplemented with 2.0 mg/L PRL for the indicated period of time. Changes in IDH1 mRNA levels were measured by semi quantitative RT-PCR. (A) Arrows indicate IDH1 or control 18S ribosomal RNA. (B) Data represent the means ± SEMs of IDH1/18S arbitrary units from 2 replicate experiments performed in triplicate (*P < 0.05).
mRNA levels. The CLAmix did not influence basal IDH1 expression, but it abrogated the stimulatory effects of PRL on IDH1 transcripts (Fig. 3A, B). The results of real-time PCR measurements confirmed that the cotreatment with the t10, c12 CLA, but not the c9, t11 CLA isomer, reduced PRL-induced IDH1 mRNA expression (Fig. 3C). Next, we investigated by western blotting the effects of the CLAmix, c9, t11 CLA, and t10, c12 CLA isomer on IDH1 protein levels. The cotreatments with the CLAmix and the t10, c12 CLA isomer reduced PRL-induced IDH1 protein expression (Fig. 4). Taken together, these results suggest that PRL stimulates a rapid increase in IDH1 mRNA and protein, whereas these effects are abrogated by selected CLA isomers.

**Discussion**

In recent years, a key role for IDH1 in lipid metabolism has been proposed (17). IDH1 catalyzes the decarboxylation of isocitrate in the cytosol and utilizes NADP\(^+\) as a cofactor to generate the reducing equivalent NADPH, which is required for fatty acid and cholesterol synthesis (21,22). It has been documented that in rat liver the enzymatic activity of IDH1 was 16- and 18-fold higher, respectively, than that of the glucose-6-phosphate and malate dehydrogenase (23), which are 2 enzymes that generate NADPH in the cytosol. Results of a study with normal subjects and obese patients have shown that IDH1 participates in fatty acid synthesis directly through the backward pathway of the Krebs cycle (24). Koh et al. (17) reported that overexpression of IDH1 by stable transfection of IDH1 cDNA stimulates adipogenesis of 3T3-L1 adipocytes and that transgenic mice overexpressing IDH1 exhibit fatty liver, hyperlipidemia, and obesity (17).

Because IDH1 may play an important role in lipid metabolism, it is important to know how the IDH1 gene is regulated. The enzymatic activity of IDH1 has been shown to increase dramatically in early lactation in bovine mammary gland (16). It has been suggested that IDH1 is the primary source of NADPH for de novo fatty acid synthesis in the bovine mammary gland due to the fact that the activities of other NADPH-producing pathways are much lower (15). We have previously reported that treatment with PRL for 72 h elicited a significant increase in IDH1 expression in a bovine mammary epithelial cell line.
The molecular mechanisms of CLA action on IDH1 gene expression require further investigation.

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Literature Cited

18. Liu W, Capuco AV, Romagnolo DF. Expression of cytosolic NADP+-dependent isocitrate dehydrogenase in bovine mammary epithelium: